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A Novel Strategy for Plant Virus Resistance Using Artificial miRNA

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ABSTRACT

Plant micro-RNAs (miRNAs) are 21-nt-long small RNAs that regulate the abundance of target mRNAs by guiding cleavage at the sequence complementary region. We found that DNA sequences encoding Arabidopsis pre-miRNAs can be expressed from a 35S promoter in transgenic plants to produce mature miRNAs. Moreover, sequences in pre-miRNAs can be modified to produce artificial miRNAs (amiRNAs) of a pre-determined sequence. Here, we describe a novel strategy to express amiRNA designed to target and degrade offending viral RNAs thereby conferring specific resistance. The amiRNA-mediated approach should have broad applicability for engineering multiple virus resistance in crop plants.

INTRODUCTION

Viruses are among the most important groups of plant pathogens in agriculture world-wide. Plant viral diseases inflict serious economic losses in major crops by

reducing yield and compromising quality (Kang et al., 2005). Crops can be protected from virus infection by resistance encoded by genes present in existing germplasms. Quite a number of dominant resistance genes (R gene) e.g. *Ry* for resistance to *Potato virus Y* (PVY) in potato (Kang et al., 2005); *Rx1* and *Rx2* for *Potato virus X* (PVX) in potato (Bendahmane et al., 1999); S*w5* for resistance to *Tomato spotted wilt virus* (TSWV) in tomato (Brommonschenkel et al., 2000), etc, have been identified. Some of these dominant genes have been used to confer virus resistance by transgenic approach. Other than dominant R genes, natural recessive resistance genes have also been reported. Several host genes, e.g. the eukaryotic initiation factor (iso) 4E (eIF4E iso), are known to be required for plant viruses to complete its infection cycle in host cells. Mutant plants with deficiency in one of these genes display resistance to potyvirus (Duprat et al., 2002).

The successful incorporation of foreign DNAs into plant cells and the generation of transformed cells into transgenic plants in 1983 opened up new opportunities to use transgenic technologies for the development of virus resistance in plants. During the ensuing two decades, pathogen-derived resistance (Sanford and Johnston, 1985) has been broadly used to genetically modify crop plants for virus tolerance/resistance world-wide. The availability of such virus-resistant crop plants promises to generate enormous economic benefit for agriculture. Nevertheless, concerns have been raised concerning possible ecological impacts of large scale and long term use of such transgenic plants in the field. In this article, we describe a novel strategy to genetically engineer virus resistance using artificial miRNA (amiRNA) technology which may mitigate several of these concerns.

Pathogen-derived Resistance

The concept of pathogen-derived resistance was first propounded by Sanford and Johnston (1985) who suggested using a portion of a pathogen's own genetic material for host defense against the pathogen itself. The underlying rationale was that certain pathogen-derived molecules (RNAs or proteins) may be critical for viral pathogenesis. Non-functional forms of such molecules could act in a dominant negative manner and interfere with virus replication, assembly or movement. Therefore, expression of such dominant negative forms of the pathogen-derived molecule in host cells may confer virus resistance in the expressing host (Sanford and Johnston, 1985).

The feasibility of pathogen-derived resistance was first examined by the expression of the *Tobacco mosaic virus* (TMV) coat protein (CP) gene in tobacco plants (Abel et al., 1986). When challenged with TMV, transgenic plants expressing the TMV CP either did not display symptoms of TMV infection or showed a delay in symptom development. The use of CP expression to confer resistance was quickly verified for several viruses. This type of pathogen-derived resistance is generally referred to as protein-mediated resistance or CP-mediated resistance. In earlier experiments, the prevailing notion was that the virus resistance level is directly related to the CP expression level of transgenic lines.

Further investigations by several laboratories, however, led to the surprising finding that that some transgenic lines with high virus resistance levels in fact did not express any viral CP. Moreover, the CP RNA level was very low or even not detectable in these resistant plants. Subsequent work clarified this apparent discrepancy of non-expressing transgenic plants with virus resistance. We now know

that these transgenic lines were resistant to virus because the expressed CP mRNA triggered post-transcriptional gene silencing (PTGS) and provided RNA-mediated resistance to virus by the siRNA pathway.

In addition to CP mRNA, RNA-mediated virus resistance can be brought about by expression of satellite RNA, defective interfering (DI) RNA or even noncoding region of viral genome RNAs which compete and interfere with virus replication (Baulcombe, 1996). This type of resistance can also be accomplished by expression of viral sequences in the sense or antisense orientation (Smith et al., 1994; Waterhouse et al., 1998) or in double-stranded forms (Helliwell and Waterhouse, 2003). In all these cases, expression triggers degradation of both the transgene RNA and the corresponding viral RNA via the siRNA pathway.

The siRNA Pathway

The siRNA pathway targets double-stranded (ds) RNA for degradation by DICER-like proteins (DCLs) in a sequence-specific manner through the production of siRNA. Whereas DCL2 cleaves dsRNAs from replicating viruses (Xie et al., 2004), DCL3 cleaves dsRNAs derived from endogenous transcripts through the activity of RDR2 and RDR6 (Dalmay et al., 2001; Mourrain et al., 2000). The siRNAs produced are incorporated into RNA-induced silencing complexes (RISC), which guide cleavage of target RNAs. In RISC, siRNAs mediate sequence-specific binding and cleavage of target RNAs (Baulcombe, 2004). Once cleaved, the RNA is further degraded by exonucleases in the cytoplasm. Alternatively, siRNAs are used as primers for RDR polymerase, using target RNA as a template to generate more dsRNA and produce additional siRNAs. This RDR activity expands the pool of siRNA and amplifies PTGS resulting in more potent silencing activity and effective defense against plant viruses.

Plant MicroRNA (miRNA) Genes and the miRNA Pathway

Recently, novel small RNAs, known as micro-RNAs (miRNAs), have been identified as important regulators of gene expression in both plants and animals. miRNAs are single-stranded RNAs 21 nucleotides (nt) in length, generated from processing of longer pre-miRNA precursors (Bartel, 2004) by DCL1 in Arabidopsis (Xie et al., 2004). These small RNAs are recruited to the RISC complex. Using RNA:RNA base-pairing, miRNAs direct RISC in a sequence-specific manner to down-regulate target mRNAs in one of two ways. Limited miRNA:mRNA base-pairing results in translational repression, which is the case with majority of the animal miRNAs studied so far. By contrast, most plant miRNAs show extensive base-pairing to, and guide cleavage of, their target mRNAs (Jones-Rhoades et al., 2006). So far, 117 miRNA genes have been identified in *A. thaliana* and Arabidopsis miRNAs have been shown to be important regulators of plant developmental processes (Jones-Rhoades et al., 2006).

The Use of Artificial miRNAs (amiRNAs) to Confer Virus Resistance

Previous reports have shown that alterations of several nucleotides within a miRNA 21-nt sequence do not affect its biogenesis and maturation (Guo et al., 2005; Vaucheret et al., 2004). This finding raises the possibility to modify miRNA sequence to target specific transcripts, originally not under miRNA control. In human cells, miR30 precursor has been modified to generate an amiRNA to down-regulate gene expression probably by translational inhibition (Boden et al., 2004; Dickins et al., 2005; Stegmeier et al., 2005; Zeng et al., 2002). In plants, Schwab et al. (2006) (Schwab et al., 2006) and Alvarez et al (2006) (Alvarez et al., 2006) have recently

reported the successful down-regulation of Arabidopsis gene expression by amiRNAs targeting either individual transcripts or groups of endogenous transcripts. These amiRNAs were constructed using precursors of miR164b, miR172a and miR319a as backbones.

In an independent study, we have also successfully produced several different amiRNAs using pre-miR159a and pre-miR169c as backbones (Niu et al, 2006). High levels of amiRNAs can be detected in transient expression assays using leaves of *Nicotiana benthamiana* and also in transgenic *Arabidopsis thaliana* carrying 35S-amiRNA transgenes. (Fig. 1). The essential features of the application of this technology for virus resistance are outlined in Fig. 2.

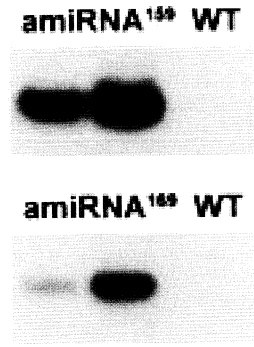

Fig.1. Transient expression of amiRNAs in *N. benthamiana* by agro-infiltration.

To explore possible biotechnological applications of the amiRNA technology, we used pre-miR159a to generate artificial pre-miRNAs^{159} (pre-amiRNAs^{159}) containing sequences complementary to genomes of two plant viruses, *Turnip yellow mosaic virus* (TYMV) and *Turnip mosaic virus* (TuMV). Transgenic lines carrying both 35S-pre-amiRNA^{159} transgenes can express the appropriate amiRNA at high levels.

Moreover, amiRNA transgenic plants showed specific resistance to either TYMV or TuMV, depending on the expression of the cognate amiRNA (Niu et al., 2006). Finally, transgenic plants that expressed both amiRNAs were resistant to both viruses and the virus resistance trait is heritable through at least 3 generations.

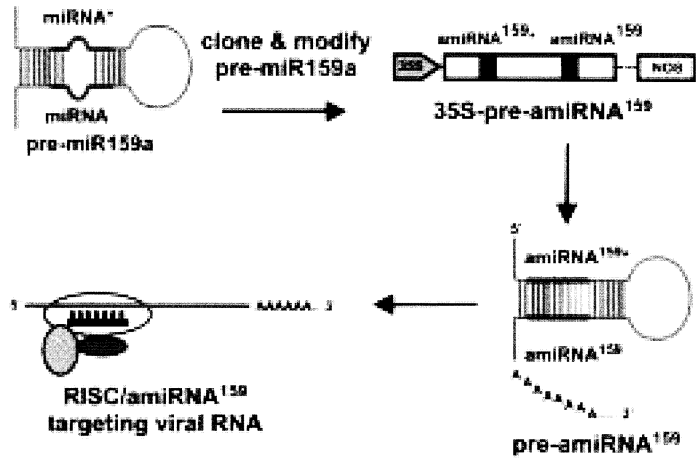

Fig.2. Engineering of amiRNA to target and degrade viral RNA.

It has been reported that virus and transgene-mediated RNA-silencing becomes attenuated at low temperatures which inhibit siRNA accumulation in insect, plant and mammalian cells (Fortier and Belote, 2000; Kameda et al., 2004; Szittya et al., 2003) This temperature sensitivity explains why siRNA-mediated virus resistance breaks down at 15 degrees C (Szittya et al., 2003). We found that miRNA accumulation was hardly affected at low temperatures. Consistent with this finding, transgenic lines expressing amiRNAs maintained their specific virus resistance even at low temperatures (Niu et al., 2006).

Comparison between Pathogen-derived Resistance and amiRNA-Mediated Resistance

Concerns have been raised regarding the large scale and long-term use in the field of transgenic plants (Tepfer, 2002). Potential risks that might occur include the following (Aaziz and Tepfer, 1999; Falk and Bruening, 1994; Hammond et al., 1999; Rubio et al., 1999; Tepfer, 2002): (1) Novel pathogens might be generated via recombination between the virus-derived transgene and non-target viruses; (2) Through possible trans-encapsidation unrelated viruses might be transmitted to host plants; (3) Transcripts expressed from the transgene with pathogen-related sequences might synergize with unrelated viruses to exacerbate symptoms; (4) There might be gene flow from transgenic pollen to weedy relatives, and (5) Transgenic plants might produce new allergens or toxic proteins. With the exception of item #4, these concerns are minimized in the case of transgenic plants engineered to express amiRNAs. We believe that the use of such amiRNA plants will improve bio-safety and reduce any possible environmental impact.

CONCLUSIONS

In this work, we describe a novel strategy to engineer specific virus resistance by the expression of appropriate amiRNA. Because of the functional conservation of miRNA action in plants this strategy should have broad applicability to other viruses as well. It should also be possible to prevent any breakdown of resistance by expressing in the same plant 2-3 amiRNAs targeting different essential regions of a virus. In addition, broad spectrum resistance to several viruses can also be obtained by co-expression of appropriately-designed multiple amiRNAs (Niu et al., 2006).

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